Pilot Grant: SaNeT-ALS

Sigma-1 receptor as a new potential therapeutic target for amyotrophic lateral sclerosis (ALS).

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**$S_1R$ (σ1) non-opioid receptor**

- **Identification of sigma receptors** (Martin et al., 1976)
- **2 subtypes of sigma receptors: $S_1R$ and $S_2R$** (Bowen et al., 1990; Giutart et al., 2004)
- **Classification of SR as a non-opioid receptor** (Quirion et al., 1992, Mitsumoto et al., 2003; Guitart et al., 2004)
- **Cloning of $S_1R$ from guinea pig liver** (Hanner et al., 1996)

A) **Cloning**

...............1857bp, 223 a.a., 25.3 KDa

B) **Homology studies**

...............yeast C8-C7 sterol isomerase 30.0% identity

C) **Tissue distribution**

...........ubiquitous; enriched in spinal cord and motor neurons (Alonso et al., 2000; Mavlyutov et al., 2010)
Molecular structure

- Transmembrane domains:

- ER retention signal:

- Ligand binding site:
Mitochondria-associated ER membranes (MAM)

S1Rs are particularly enriched in motor neurons. Mainly localized at the ER mitochondrial associated membranes (MAM) and at very low levels in post-synaptic thickenings of the neuron (Hayashi and Su, 2007; Su et al., 2010).
S1R intracellular dynamics

S1R
Inter-organelle signaling modulator:
-cell type
-microenvironment

Sun et al., 2010
S1R involvement in different pathologies

“Few agonists and antagonists of sigma receptors are currently in clinical trial for acute and chronic neurodegenerative diseases or neuropathic pain”

(Cobos et al., Neuropharmacology, 2008; Collina et al., Expert Opin Ther Pat, 2013)

Sigma-1 Receptor Ligands

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzomorphan</td>
<td>Synthesis</td>
</tr>
<tr>
<td>(+)SKF-10047</td>
<td>agonist</td>
</tr>
<tr>
<td>(+)Pentazocine</td>
<td>agonist</td>
</tr>
<tr>
<td>Synthetic compounds</td>
<td></td>
</tr>
<tr>
<td>PRE-084</td>
<td>agonist</td>
</tr>
<tr>
<td>Antidepressant</td>
<td>agonist</td>
</tr>
<tr>
<td>Neurosteroid</td>
<td>antagonist</td>
</tr>
<tr>
<td>Progesterone</td>
<td>antagonist</td>
</tr>
<tr>
<td>Antipsychotic</td>
<td>antagonist</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>antagonist</td>
</tr>
<tr>
<td>Synthetic compounds</td>
<td></td>
</tr>
<tr>
<td>NE-100</td>
<td>antagonist</td>
</tr>
</tbody>
</table>
S1R involvement in motor neuron protection

2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate PRE-084

Neuritogenic effect of PRE-084 (10 µM) on organotypic spinal cord slices

Neuroprotection mediated by PRE-084

(from Guzmán-Lenis, Navarro and Casas, Neuroscience, 2009)
S1Rs contribute to the pathogenesis of ALS:

Frontotemporal Lobar Degeneration with Motor Neuron Disorders (FTLD-MND).

Sigma Nonopiod Intracellular Receptor 1 Mutations Cause Frontotemporal Lobar Degeneration—Motor Neuron Disease

Objective: Frontotemporal lobar degeneration (FTLD) pathologically disordered inclusion bodies observed in a variety of neurodegenerative disorders. There is an urgent need to identify the causative genes in FTLD-MND. The primary objective was to identify the causative genes in Frontotemporal Lobar Degeneration with Motor Neuron Disorders (FTLD-MND). A mutational screen of candidate genes, however, performed to identify the biological role of the positive individuals and western blot studies of cell lines were performed. Results: We identified a nonpolymorphic mutation (G→C) in the Sigma Nonopiod Intracellular Receptor 1 (S1R) gene in an FTLD-MND patient. The mutation was confirmed in the patient's parents and siblings. The mutation was absent from 40 control alleles. The mutation results in an amino acid change from glutamate to glutamine (E102Q). It is predicted to lead to a change in the protein conformation and the loss of function.

Juvenile ALS

Missense mutation (G→C) in exon 2 of the S1R gene leads to the substitution of glutamine for glutamate at amino acid position 102 (E102Q).

LACK OF SIGMA-1 RECEPTOR EXACERBATES ALS PROGRESSION IN MICE

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Abstract—The function of the sigma-1 receptor (S1R) has been implicated in modulating the activity of various ion channels. In the CNS S1R is enriched in cholinergic postsynaptic densities in spinal cord motor neurons (MNs). Mutations in S1R have been found in familial cases of amyotrophic lateral sclerosis (ALS). In this study we show that a knockout of S1R in the SOD1-G93A mouse model of ALS significantly reduces longevity (end stage). Electrophysiological experiments demonstrate that MN of mice lacking S1R exhibit increased excitability. Taken together, these results suggest that S1R acts as a brake on excitability, an effect that might enhance longevity in an ALS mouse model.

Key words: sigma-1 receptor, C-terminals, ALS, motor neurons, excitability, Kv2.1.
Effect of chronic S1R agonist treatment in SOD1-G93A transgenic mice

Daily administration of PRE-084 (0.25 mg/kg i.p.) from 8 weeks of age significantly improved spinal motor function and motoneuron survival (Mancuso et al. Neurotherapeutics, 2012)

PRE-084 treatment starting at 8 weeks of age

Delayed administration of PRE-084 starting at 12 weeks of age

PRE-084 administration significantly extended SOD1 animals lifespan 16 days in females and 11 days in males.
SaNeT-ALS Pilot Grant

Our project is based on the hypothesis that sigma-1 receptor (S-1R) may represent a new therapeutic target for amyotrophic lateral sclerosis (ALS).

To test this hypothesis, we shall evaluate the effect of two S-1R agonists on motoneuron survival, motor performance and disease progression in 2 murine model of motoneuron degeneration. The first S1R agonist is an already known and well characterized molecule (PRE-084). The second is a novel, potent and selective S-1R agonist, recently synthesized in our labs (NS1Ra).

(a) Wobbler (wr) mice
(b) SOD1-hG93A mice

Mutation L967Q of the gene encoding for Vps54 subunit of GARP (Golgi-associated retrograde protein) complex.

- Symptomatic phase starting at 3 weeks of age
- Stabilization phase at 12 weeks of age

HALLMARKS
✓ Astrogliosis and microgliosis
✓ ER stress
✓ Ubiquitin and TDP-43-positive protein aggregates
✓ Mitochondrial dysfunction
✓ Vesicle trafficking defects
✓ Impaired axonal transport
✓ Cortical hyperexcitability
Increase of S1R signal and KDEL de-colocalization in motor neurons of 6 weeks-old wr mice.

S1R staining is reduced in motor neurons displaying clear signs of neuronal sufferance.
S1R staining in glial cells of symptomatic wr mice.

- Some hypertrophic astrocytes display cytoplasmatic S1R signal.

- S1R signal was detectable in ramified and reactive microglial cells.
Effect of PRE-084 on locomotor performance and motor neuron survival.

PRE084 (0.25 mg/kg i.p., 3 day/week) treated wr mice displayed beneficial effects on motor performance in comparison with vehicle-treated mice (A,B). Morphometric histological analyses conducted on the cervical spinal cord, anterior horn, of 12 weeks-old treated mice showed a significant increase in the number of motor neurons vs vehicle-treated mice (C).
S1R signal in motor neurons of 12 weeks-old wr mice
Effect of PRE084 on gliosis in wr mice

- Reduction of reactive astrogliosis in PRE-084 treated wr mice.
- Increase in the mean number of CD11b+ microglial cells.

Number and density of GFAP+ cells (C,D) and of CD11b+ cells (E,F) in vehicle-(squares) and PRE-084-treated (triangles) wobbler (wr) mice. Data are the mean ± SD of n=6 mice for each experimental group.*, § =p<0.05, **=p<0.01, unpaired Student’s “t” test vs vehicle-treated group.
PRE-084 treatment increases CD68+ reactive microglial/macrophage phenotype in the white matter.

CD68+ cells are condensed in close proximity of GFAP+ astrocytes
Many round-shaped peripheral cells are both CD68+ and CD206+.

(A-F) Laser scanning confocal photomicrographs of CD68 (green, A, D), CD206 (red, B, E) and Hoechst (blue) nuclear staining in ventral horn cervical spinal cord of 12 weeks-old vehicle (veh)- and PRE-084-treated wr mice. Dashed lines in A-D highlight the boundaries between gray (g.m.) and white (w.m.) matter. CD206+ cells are mainly detectable at the boundaries of the white matter (arrowheads in B, C and E, F). CD206+ cell staining increases in PRE-084-treated wr mice. As highlighted at higher magnifications and through orthogonal projection (G), many round-shaped peripheral cells are both CD68+ and CD206+ (arrowheads in G); CD206 signal is also detectable in some highly stained amoeboid-like CD68+ cells throughout the parenchima (arrows in G). Dashed lines in G highlight the boundaries of the spinal cord section. Scale bar in (F) applies to (A-F) = 20 µm. Scale bar in G = 10 µm.
HIGHLIGHTS

1) Alterations of Sigma-1 receptor (S1R) in wobbler mouse model of Motor Neuron Disease

2) Treatment with a S1R agonist improves motor neuron survival and locomotor performance in wobbler mice

3) S1R is a therapeutic target not only in SOD1-linked but also in non-SOD1-linked ALS

4) Modulation of neuroinflammation may be part of the mechanisms involved in S1R agonist-mediated neuroprotection
* Scale up synthesis of novel and selective S1R agonist (NS1Ra)  
  (Lab. Medicinal Chemistry, Dept. Drug Sciences, University of Pavia)
* Chronic treatment of SOD1-G93A mice  
  (AriSLA Facility, M.Negri Institute for Pharmacological Research)

1st group (daily treatment for 4 weeks)
A-vehicle (saline i.p.)
B-PRE-084 (0.25 mg/Kg, i.p.)
C-PRE-084 (0.25mg/Kg, i.p.) + Riluzole  
  (25mg/Kg in the drinking water)
D-NS1Ra dose 1 (0.01 mg/Kg, i.p.)
E-NS1Ra dose 2 (0.1 mg/Kg, i.p.)

2nd group (treatment until survival)
  -vehicle
  -PRE-084
  -PRE-084+ Riluzole
  -NS1Ra dose 1
  -NSRa dose 2

...........ending June 2013
**NSC-34D**

Culturing in low-serum medium containing araC improves the homogeneity of NSC-34D cultures, leaving only differentiated cells displaying long neurites.

Thapsigargin-induced caspase 3/7 activity

<table>
<thead>
<tr>
<th>Drug</th>
<th>Caspase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
<td>0.5 ± 0.02</td>
</tr>
<tr>
<td>T</td>
<td>1.2 ± 0.03</td>
</tr>
<tr>
<td>T+NE</td>
<td>0.8 ± 0.01</td>
</tr>
<tr>
<td>T+PRE+RIL</td>
<td>0.6 ± 0.00</td>
</tr>
<tr>
<td>T+RIL</td>
<td>1.0 ± 0.04</td>
</tr>
<tr>
<td>T+PRE</td>
<td>0.7 ± 0.02</td>
</tr>
</tbody>
</table>

Mean ± SD of N=4 different experiments performed in triplicate. * = p<0.02, paired Student’s “t” test.

- pre-exposure for 8 h with thapsigargin (T, 0.5 µM).
- PRE-084 (PRE, 10 µM); riluzole (RIL, 10 µM); combined PRE-084 + riluzole (PRE+RIL) or PRE-084 + NE-100 (NE, 3 µM) were added. Cells were incubated for further 48h.
S1R agonist potentiation of NGF-induced neurite outgrowth

**NS1Ra potentiation of NGF-induced neurite outgrowth**

**PRE-084 potentiation of NGF-induced neurite outgrowth**
Effect of the 4-week drug treatment on symptomatic SOD1-G93A transgenic mice

<table>
<thead>
<tr>
<th></th>
<th>Rota-Rod</th>
<th>Grip Strenght</th>
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<tbody>
<tr>
<td></td>
<td>3 weeks of</td>
<td>3 weeks of</td>
</tr>
<tr>
<td></td>
<td>treatment</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>AV</td>
<td>AV</td>
</tr>
<tr>
<td>Vehicle vs NS1Ra dose 1</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Vehicle vs NS1Ra dose 2</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>dose 1 vs dose 2</td>
<td>yes 0.041</td>
<td>yes 0.027</td>
</tr>
<tr>
<td>4 weeks of treatment</td>
<td></td>
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</tr>
<tr>
<td>Vehicle vs NS1Ra dose 1</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Vehicle vs NS1Ra dose 2</td>
<td>yes 0.007</td>
<td>yes 0.006</td>
</tr>
<tr>
<td>dose 1 vs dose 2</td>
<td>yes 0.011</td>
<td></td>
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</tbody>
</table>

Tukey's test for multiple comparisons

- **Rota-Rod**
  - 3 weeks of treatment: significant, AV
  - Vehicle vs NS1Ra dose 1: no
  - Vehicle vs NS1Ra dose 2: no
  - dose 1 vs dose 2: yes, 0.041

- **Grip Strenght**
  - 3 weeks of treatment: significant, AV
  - Vehicle vs NS1Ra dose 1: no
  - Vehicle vs NS1Ra dose 2: no
  - dose 1 vs dose 2: yes, 0.027

- 4 weeks of treatment
  - Vehicle vs NS1Ra dose 1: no
  - Vehicle vs NS1Ra dose 2: no
  - dose 1 vs dose 2: yes, 0.006
Highlights

• The novel compound seems to be effective when administered at the symptomatic stage of the disease

• The behavioral effect are apparent shortly after the beginning of treatment

However, before drawing conclusions we should wait for the results of treatment until survival ... and focus on the mechanism underlying S1R mediated motor neuron protection.
Thanks to...

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SaNeT-ALS – Call 2011
Ente adottante Nova Coop
Compared to ‘healthy’ control conditions (A), C boutons appear to be reduced in number in spinal cord injury (B) and enlarged in amyotrophic lateral sclerosis (ALS; C). Decreased C bouton numbers in spinal cord injury should reduce motor neuron excitability and the frequency of motor neuron output (B, bottom trace), perhaps contributing to overall motor dysfunction. Conversely, enlarged C boutons in ALS should lead to greater motor neuron excitability, which might contribute to excitotoxic disease mechanisms.

(from Mavllyutov et al., 2010; Witts et al., 2013)